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(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Michael HOUGHTON et al.

Application No.: 10/438,313

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For: HEPATITIS C VIRUS PROTEASE

Confirmation No.: 4307

Art Unit: 1652

Examiner: R. Prouty

DECLARATION OF AMY J. WEINER UNDER 37 C.F.R. § 1.132

I, Amy J. Weiner, declare and affirm that:

1. I am currently Director of Research, Vaccines and Antivirals at Chiron Corporation, Emeryville, California.

2. I have a Ph.D. in Molecular, Cellular and Development Biology from Indiana University. Since 1984 I have been involved in research on hepatitis viruses. A copy of my curriculum vitae and list of publications is attached.

3. I am not an inventor of U.S. Patent No. 5,371,017.

4. I have read the above referenced U.S. Patent No. 5,371,017, and I understand the subject matter contained therein. I am qualified to comment on what one of ordinary skill in the art would understand from reviewing the disclosure of this patent and the publications referred to in my declaration.

U.S. Patent No. 5,371,017 discloses a HCV protease

5. I have reviewed U.S. Patent No. 5,371,017 ("the '017 patent") and believe that one of skill in the art would understand that the specification discloses a Hepatitis C Virus (HCV) protease.

6. The '017 patent specification describes a HCV protease. The specification states that: "[t]he term 'HCV protease' refers to an enzyme derived from HCV which exhibits proteolytic activity, specifically the polypeptide encoded in the NS3 domain of the HCV genome." (Column 4, lines 17-20) An HCV protease sequence is provided in Figure 1 of the patent specification. (Column 2, lines 14-15). The specification points to a section in the NS3 domain of HCV as the key to proteolytic activity and notes that the termini of the relevant section are putative. (Column 4, line 20 through column 5, line 6). Column 8, lines 45-57 refer to an NS3 domain by analogy with the Yellow Fever Virus (a flavivirus) polyprotein. An HCV protease encoded by the NS3 domain in at least one strain of HCV is further described with reference to a 202 amino acid protease sequence from SEQ ID NO: 1 (claim 12) in column 4, lines 17-41 (SEQ ID NO: 65; claim 15). HCV protease activity associated with a 299 amino acid HCV polypeptide encoded by SEQ ID NOS: 66 and 68 and specified in claims 6 and 14 are described in Example 5. (col. 20, lines 56-58). HCV protease activity associated with a 686 amino acid HCV polypeptide shown in Figure 1 and encoded by SEQ ID NO: 70, and specified in claim 2 is described in Examples 4 and 5. (col. 19, lines 11-16; and col. 20 lines 33-42).

7. A protease activity is characterized in Example 5 (col. 20, line 21 – col. 21, line 12) which shows self cleavage of hSOD–HCV protease fusion proteins expressed in *E. coli*. Col. 20, lines 34-36 states that "[t]he results indicated the occurrence of cleavage, as no full length product (theoretical M_r = 93 kDa) was evident on the ge[illegible]."

8. Example 4 (col. 19, lines 1 – 46) describes the amino acids of HCV protease encoded by each fusion protein.

9. The P190 fusion product encoding amino acids 1-199 of the HCV protease (col. 19, lines 25-27) showed no protease cleavage activity (col. 21, lines 6-9).

10. P300 which includes amino acids 1-299 of HCV protease (col. 19, lines 34-36; SEQ ID NOS: 66 and 68) indicated occurrence of cleavage (col. 20, lines 56-58).

11. P500 comprising amino acids 1-513 of Fig. 1 (col. 19, lines 43-46) indicated occurrence of cleavage (col. 20 lines 49-51).

12. The fusion protein ("P600") encoded by the vector cf1SODp600 which includes amino acids 1-686 of Fig. 1 (SEQ ID NO: 70) also showed proteolytic activity. (col. 19, lines 11-16; and col. 20 lines 33-42).

13. The specification concludes that "the minimum essential sequence for HCV protease extends to the region between amino acids 199 and 299." (col. 21, lines 9-11).

14. One of skill in the art would understand from reviewing the '017 patent that a protease activity associated with a specific segment of HCV polyprotein is disclosed in the specification.

U.S. Patent No. 5,371,017 discloses a substrate for the HCV protease

15. A peptide substrate for a HCV protease is also disclosed in the specification. The protease activity described in Examples 5 (A), (B), and (C) was observed through self-cleavage of a hSOD-HCV fusion protein wherein the HCV peptide portion corresponded to amino acids 1-686 of Fig. 1 and various truncations thereof. Observance of specific cleavage within the NS3 region of HCV is described in every instance where protease activity was observed. For example, "34 kDa band correspond[ing] to the hSOD partner (about 20 kDa) with a portion of the NS3

domain" was observed in each case with the P600, P300 and P500 fusion proteins of NS3 fused to a hSOD leader.

The protease activity described in the '017 patent is not bacterial

16. One of skill in the art would understand that the lack of protease cleavage activity upon mutation of the active site residue within the HCV protease sequence disclosed in the specification, indicates that the protease activity is caused by HCV sequences and not by host bacterial enzymes. (*see* Hijikata et al. (1993)).

17. Example 10 of the specification provides a prophetic description of protease assays designed to show HCV protease activity associated with the claimed sequences by use of a pGEM-3Z/Yellow Fever Leader vector for in vitro expression of HCV protease. In vitro transcription and translation of the clone HCV protease using transcription and translation systems from Promega are disclosed in Example 11, column 23 of the specification.

18. These experiments were subsequently carried out and results reported by Eckart *et al.* in Biochem. Biophys. Res. Commun. 192:399-406 (1993). I have reviewed and understand the contents of the Eckart *et al.* publication.

19. Eckart *et al.* discloses that expression in a rabbit reticulocyte system of a pGEM-3YPN vector containing 5' truncated NS2 and 3' truncated NS3 fragment of HCV (corresponding to HCV amino acids 840-1619) showed protease activity encoded by this region. (Eckart p. 403 and Fig. 2). The Eckart fragment expressed in the rabbit reticulocyte system corresponds to HCV amino acids 840-1619, while the cf1SODp600 described in Examples 4 and 5 of the specification corresponds to amino acids 946-1630 of HCV.

20. When the NS2'-NS3' fragment was tested with a mutation at Ser-1165, Eckart found that "[i]dentical polypeptide profiles [of the protease products] were observed in translation of both wild type and mutant RNA templates (Fig. 2) indicating that the NS2/NS3 cleavage occurs inefficiently but independently of Ser_{1165}." (Eckart, p. 403).

21. The Eckart paper confirms the '017 patent's prophetic example of a protease encoded by the 5' truncated NS2 and 3' truncated NS3 fragment of HCV or amino acids 946-1630 of HCV. Since these experiments were conducted in a mammalian rabbit reticulocyte system, one of skill in the art would understand that the protease activity could not possibly be bacterial.

The HCV protease activity described in the '017 patent is attributable to the NS2/NS3 protease

22. I have reviewed and understand the following publications discussed in the below paragraphs: Pallaoro et al. J. Virol. 9939-9946 (2001); Hijikata et al. J. Virol 67(8):4665-4675 (1993); Grakoui et al. Proc. Natl. Acad. Sci. (USA) 90:10583-10587 (1993); Santolini et al. J. Virol. 69:7461–7471. (1995); Reed et al. J. Virol. 69:4127-4136 (1995); Pieroni et al. J. Virol. 71(9):6373-6080 (1997); and Thibeault et al. J. Biol. Chem. 276(49):46678-46684 (2001).

23. A serine protease encoded in the N-terminal region of the NS3 domain is responsible for cleavage of the HCV polyprotein at sites downstream of the NS3 gene. The NS2 protein extends from amino acid 810 to amino acid 1026, and cleavage at the NS2-NS3 junction involves a second viral protease which comprises part of the NS2 region and the entire NS3 domain. (*see* Grakoui et al. (Proc. Natl. Acad. Sci. (USA) 90:10583-10587 (1993)).

24. The fusion proteins expressed in Example 5, comprise 1-151 amino acids from hSOD (human superoxide dismutase gene) and amino acids 946-1630 of HCV (corresponding to amino acids 1-686 of Figure 1) and C-terminal truncations therefrom. *See* col. 18, lines 19-22.

25. Example 4 reports how the clones used in Example 5 were constructed. The largest construct used was p600. According to Example 4, this construct encompassed amino acids 946-1630 of HCV. Grakoui et al. (Proc. Natl. Acad. Sci. (USA) 90:10583-10587 (1993)) reported that NS3 consists of amino acids 1027-1657 of HCV. A comparison of the HCV amino acids included in p600 with the NS3/NS4 boundaries reported in Grakoui shows that the downstream ends of the Example 5 constructs stop short of the NS3/NS4 boundary, which occurs after amino acid 1657, while their upstream ends include what is now understood to be a portion of NS2.

26. The putative cleavage site for the NS2/3 protease is between Leu-1026 and Ala-1027. (Grakoui, Proc. Natl. Acad. Sci. (USA) 1993, p. 10584). These residues correspond to amino acids 81 and 82 of the sequence of Figure 1. Fused with a 151 amino acid hSOD, these are expected to produce a fragment of 232 amino acids upon NS2/3 cleavage. In Example 5 of the specification, P600, P500 and P300 fusion proteins resulted in a 34 kDa fragment which corresponds to a predicted 232 amino acid cleavage product. P190 which is inactive, does not produce a 34 kDa product but only a 40kDa protein corresponding to a presumably uncleaved product.

27. Subsequent characterization of the NS2/3 protease has shown it to be a cysteine protease and site-directed mutagenesis studies have identified His-952 and Cys-993, numbered according to their location within the HCV polyprotein, as essential for its activity. (Pallaoro et al. J. Virol. 9939-9946 (2001); Hijikata et al. J. Virol 67(8):4665-4675 (1993); Grakoui et al. Proc. Natl. Acad. Sci. (USA) 90:10583-10587 (1993)). His-952 corresponds to amino acid 7 of Figure 1 and Cys-993 corresponds to amino acid 48 of the sequence of Fig. 1. Thus the amino acid residues corresponding to the two residues essential for NS2/3 protease activity are within the sequence of Figure 1 and the constructs of Example 5.

28. The NS 2/3 viral protease includes most of the NS2 region and part of the N-terminus of NS3 serine protease domain, amino acids 849 to 1237. The catalytic activity of the serine protease is not required for the NS2–NS3 cleavage (Hijikata et al. (1993); Grakoui et al. (1993)).

29. Hijikata states "[t]he catalytic activities of these two proteinases are separable, because some mutants, such as S1165A and H952A, retained only one of these activities. However, the regions required for detection of these activities in the HCV precursor polyprotein overlapped." (p. 4673). The Eckart paper discussed above also confirms that mutation of the serine-1165 residue which inactivates NS3 serine protease, does not affect the activity of the NS2/3 protease.

30. The NS3 portion of the viral enzyme cannot be substituted by other fragments of the HCV polypeptide. (Santolini et al. J. Virol. 69:7461–7471. (1995)). Santolini shows that while a NS2-NS3 polypeptide extending to HCV residue 1237 is active for NS2/3 protease activity, a polypeptide extending to residue 1137 is not. (*see* Fig. 1B of Santolini). The results of Example 5 shows that fusion protein P190 extending to amino acid 1145 is inactive while fusion protein P300 extending to amino acid 1245 is active. Thus Example 5 corresponds to the observations of Santolini about the requirement for the minimum length of NS3 sequence for NS2/3 protease activity.

31. The experiments in Hijikata, Grakoui and Santolini references are fundamentally different from Example 5, as they use *in vitro* translated proteins that do not have (or have fewer than 10) amino acids upstream of the N-terminal NS2 truncation fragment. In contrast to the lack of (or very few) amino acids upstream of the sequences of Hijikata, Grakoui and Santolini, a 151 amino acid hSOD leader peptide is attached to the N-terminus of the fusion proteins expressed in Example 5.

32. The NS 2/3 cleavage activity is affected by microsomal membranes (Santolini) and detergents (Pieroni). Activity is inhibited by mutations that perturb local conformation and suggest the importance of correct folding of the NS 2/3 polypeptide for proper cleavage activity. (Pieroni, at 6373; *see also* Hijikata; Grakoui (Proc. Natl. Acad. Sci. (USA), 1993); Reed et al. J. Virol. 69:4127-4136 (1995)). The NS2 protein extends from HCV residue 810, and the NS2/3 protease extends between HCV residues 810 and 1206 (*Id.*). However, the references discussed above show that it is possible to delete numerous residues upstream of His-952 and Cys-993 and retain activity. Thibeault *et al.*(J. Biol. Chem. 276(49):46678-46684 (2001)) have been able to express the NS2/3 protease in bacteria and observed significant protease activity in NS2/3 peptides extending from residue 904, and lesser but detectable activity in peptides containing residues 915-1206. (*see* Fig. 2C). Thibeault's expression system only adds 10 upstream amino acids (*Id.* at p. 46679).

33. There is no demonstrated requirement for specific residues at specific positions upstream of His-952 and Cys-993. As few as about 40 upstream residues demonstrate significant activity (Thibeault).

34. Therefore, one of skill in the art would understand that the fusion of a 151 amino acid fragment from hSOD to the 946-1630 HCV fragment was sufficient to generate NS2/3 protease activity in the fusion proteins of Example 4 as evidenced by the results of Example 5.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Executed this 1st th day of August, 2005

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EDUCATION

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1984-present Chiron Corporation
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